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Evaluation of Multiplex Type-Specific Real-Time PCR Assays Using the LightCycler and Joint Biological Agent Identification and Diagnostic System Platforms for Detection and Quantitation of Adult Human Respiratory Adenoviruses▽

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Every year, thousands of basic military trainees in each service of the U.S. Armed Forces experience acute respiratory disease. The majority of this disease burden results from infection with human adenoviruses. We designed single- and multiplex assays that detect and discriminate adenovirus types B3, E4, B7, B11, B14, and B21. A total of 116 oropharyngeal swab specimens obtained from patients at the Naval Health Research Center were used to validate the new assays. Type-specific singleplex assays were designed and used independently to successfully identify 94 representative patient specimens. The lower limits of detection for our singleplex real-time PCR assays were calculated to be 50, 500, 500, 50, 50, and 50 genomic copies per reaction for human adenovirus type B3 (HAdV-B3), HAdV-E4, HAdV-B7, HAdV-B11, HAdV-B14, and HAdV-B21, respectively. These were then multiplexed to increase efficiency and tested against singleplex assays using titrated controls. The HAdV-B3/B11 and HAdV-E4/B7 multiplex assays were as sensitive and specific as they were individually. The HAdV-B14/B21 multiplex assay was not as efficient at detecting HAdV-B14 as the singleplex assay. Interestingly, a statistically significant difference was found between the viral loads of HAdV-B14 and those of HAdV-B3, -E4, -B7, and -B21 ($P < 0.001$). The assays did not cross-react with other adenoviruses, influenza virus, respiratory syncytial virus, or respiratory disease-causing bacteria. These assays have the potential to be useful as clinical diagnostic tools for the detection of HAdV infection in adult populations.

Human adenoviruses (HAdVs) were first associated with clinical illness among military trainees with respiratory disease in the early 1950s (6, 17). HAdVs were the first respiratory viruses to be isolated and characterized. Epidemiological studies showed that adenoviruses are a primary cause of acute respiratory disease (ARD) among military recruits (3, 5) and are a common cause of epidemic respiratory illness in crowded adult civilian populations (18, 21). The diverse human pathogens in the genus *Mastadenovirus* are categorized into 54 types of adenoviruses. The 54 known types have been grouped into seven species (A to G), based on their immunochemical responses, nucleic acid characteristics, hexon and fiber protein characteristics, biological properties, and phylogenetic analysis (7, 24). They are associated with a broad range of symptoms, including those associated with ARD, conjunctivitis, genitourinary infections, and gastroenteritis, and specific types of adenovirus are associated with specific types of disease (18, 21).

Adenoviruses of species B, C, and E are associated with ARD. Species C types, including HAdV type C1 (HAdV-C1), -C2, -C5, and -C6, are common causes of endemic respiratory illness in pediatric populations, and the majority of adults have acquired immunity to these types (8, 16). These may generate asymptomatic carrier-state infections that last into young adulthood, resulting in viral shedding detectable by PCR. Diverse species B types, including HAdV-B3, -B7, -B11, -B14, and -B21, and the sole species E type, HAdV-E4, cause epidemic outbreaks of ARD and conjunctivitis among adults and children (14, 22). These are also associated with essentially continuous outbreaks among unvaccinated military recruits (14, 23). Symptoms range from those associated with mild ARD to severe pneumonia, occasionally resulting in death, even among these otherwise healthy young adults (1, 4, 11, 19). Among U.S. military trainees alone, HAdVs are estimated to cause 22,000 cases of ARD that is severe enough to require medical attention, delaying training schedules, decreasing the quality of life of trainees, and costing the U.S. government $40 million annually (19).

The impact and distribution of different types varies over time and can be drastically altered through use of type-specific vaccines (14, 22, 23). An effective vaccine for HAdV-B7 and HAdV-E4 was used in the United States between 1971 and 1999, when production ceased and it became unavailable. Currently, a replacement vaccine is in clinical trials and may soon be available (19, 22), but this vaccine will be limited to use for types 4 and 7. The recent emergence in the United States of a new type, HAdV-B14a (which had not previously been seen in the Western Hemisphere), caused severe outbreaks among both military trainees and civilians and was the subject of several specific outbreak investigations (2, 12, 22). Similarly, in other regions and other circumstances, the severity, attack rates, specific symptoms, and relative risk of fatality may vary

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14. ABSTRACT

Every year, thousands of basic military trainees in each service of the U.S. Armed Forces experience acute respiratory disease. The majority of this disease burden results from infection with human adenoviruses. We designed single- and multiplex assays that detect and discriminate adenovirus types B3, E4, B7, B11, B14, and B21. A total of 116 oropharyngeal swab specimens obtained from patients at the Naval Health Research Center were used to validate the new assays. Type-specific singleplex assays were designed and used independently to successfully identify 94 representative patient specimens. The lower limits of detection for our singleplex real-time PCR assays were calculated to be 50, 500, 500, 50, 50, and 50 genomic copies per reaction for human adenovirus type B3 (HAdV-B3), HAdV-E4, HAdV-B7, HAdVB11, HAdV-B14, and HAdV-B21, respectively. These were then multiplexed to increase efficiency and tested against singleplex assays using titrated controls. The HAdV-B3/B11 and HAdV-E4/B7 multiplex assays were as sensitive and specific as they were individually. The HAdV-B14/B21 multiplex assay was not as efficient at detecting HAdV-B14 as the singleplex assay. Interestingly, a statistically significant difference was found between the viral loads of HAdV-B14 and those of HAdV-B3, -E4, -B7, and -B21 ($P < 0.001$). The assays did not cross-react with other adenoviruses, influenza virus, respiratory syncytial virus, or respiratory disease-causing bacteria. These assays have the potential to be useful as clinical diagnostic tools for the detection of HAdV infection in adult populations.

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greatly between types. It is clearly of significant public health interest to track adult respiratory adenoviruses in a type-specific manner.

Historically, adenoviruses were detected by tissue culture methods and discriminated by type-specific serum neutralization methods (13). However, traditional (probeless) PCR assays have since replaced these methods, owing to their greater speed, their significantly lower cost, and the decline in availability of type-specific antisera (15). A variety of reliable PCR assays have been developed and used, including species-specific (26) and type-specific (25) tests. Universal PCR assays paired with sequence analysis have been used to provide a truly comprehensive detection and discrimination method for all HAdV types (20). Real-time (probe-based) PCR platforms now offer even greater efficiency, improved sensitivity and specificity, and the added information value resulting from quantitative analysis of viral titers (9).

In this study, we developed a series of single- and multiplexed real-time PCR assays for both the LightCycler and the military Joint Biological Agent Identification and Diagnostic System (JBAIDS) platforms which can detect and discriminate all HAdVs implicated in adult epidemic ARD in the United States, including HAdV-B3, -E4, -B7, -B11, -B14, and -B21. Combined, these tools offer a rapid, high-throughput method for universal detection, discrimination, and quantitation of HAdVs in uncultured throat swab specimens. These assays will allow for much more rapid outbreak assessment and, if validated as in-house diagnostic assays, more rapid individual and public health responses.

MATERIALS AND METHODS

Clinical samples. The Institutional Review Board-approved inclusion criteria for consented subjects enrolled through the Naval Health Research Center (NHRC) febrile respiratory illness surveillance system were military recruits reporting for medical care with respiratory symptoms and a fever of ≥38°C. Samples collected through the NHRC program were oropharyngeal swabs resuspended in Copan viral transport medium (VTM), subsequently frozen at −80°C, and transported on dry ice for testing. Aliquots used in this study were subjected to one freeze-thaw cycle in the process of aliquoting them upon receipt at NHRC and one further freeze-thaw cycle when aliquoted for shipment to Travis Air Force Base (AFB). These samples were otherwise unprocessed, collected, and transported under Clinical Laboratory Improvement Act/Clinical Laboratory Improvement Program compliant diagnostic protocols.

Specimen processing. Throat swabs and cultured isolates were processed in a biosafety level 2 hood. Nucleic acid was extracted from 175-μl aliquots using the MagNA Pure LC DNA isolation kit I (Roche, Indianapolis, IN), according to the manufacturer's recommendations for the MagNA Pure LC automated nucleic acid extraction system. These samples were scored negative if no amplicon was acquired through conventional PCR for HAdV-B3, -E4, -B7, -B11, -B14, or -B21. Adenovirus strains for HAdV-B14 (VR-15), -B3 (VR-3), -E4 (VR-1572), -B7 (VR-7), -B11 (VR-12), -B21 (VR-256), *Haemophilus influenzae*, influenza A virus (ATCC VR-96), human rhinovirus 14 (ATCC VR-284), human parainfluenza virus 2 (ATCC VR-92), human respiratory syncytial virus (ATCC VR-26), *Chlamydophila pneumoniae* (ATCC 53592), *Escherichia coli*, *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 97), *Mycoplasma pneumoniae*, and *Legionella pneumophila* (ATCC 33152) were acquired from the American Type Culture Collection (ATCC; Manassas, VA). Genomic DNA from each adenovirus strain was quantitated by calculating the number of genomes based on the A_{260} reading using the NanoDrop 8000 (Thermo Scientific, Wilmington, DE).

Quantitative real-time PCR. The primers used to detect the HAdV-B3 amplicon were prAdV3-FibF, 5′-CGTATCCATTTGTCCTTCCTAAT-3′, and prAdV3-FibR, 5′-TAACATAGGATGTGCGACTATCT-3′. The fluorescence resonance energy transfer (FRET) probes used to detect the HAdV-B3 fiber gene have the following sequences: prbAdV3-Flr, GCCCTTTTTCCGTTGGAAGTTA CTG-Flr, and prbAdV3-640, LC-Red-640-ATGCTTAATAAACGCC-Phos. The primers used to detect the HAdV-E4 amplicon were prAd4-470F, 5′-ATGATAA AGGGAATATAAAGATTACCCTAA-3′, and prAd4-629R, 5′-GTACTACTGG TTCCGAACTC-3′. The FRET probes used to detect the HAdV-E4 hexon gene have the following sequences: prbAd4-FLR538, AGCAACATCAGTTGGGCTAA AGGTATAA-Flr, and prbAd4-640, LC-Red-640-TTTGAAGATGGTGCCATAG CTACAAACATTGGT-Phos. The primers used to detect the HAdV-B7 amplicon were prHAdV7-1230F, 5′-CAAATATCAAGGCATTAAACCTAGAG-3′, and prHAdV7-1396R, 5′-CATCTGGAAGGTACAAAGCC-3′. The FRET probes used to detect the HAdV-B7 hexon gene have the following sequences: prbAdV7-Fluor9, CCATAGGCAACAATCTGGCTATGGAAATTA-Flr, and prbAdV7-705-9, LC-Red-705-ATCCAAGCTAATCTTTGGAGAAGTTTTCT GTACT-Phos. The primers used to detect the HAdV-B11 amplicon were prAD11-5F, 5′-GTGGATTGCAGAAGGTGTA-3′, and prAD11-5R, 5′-TTAC TTTCTTCATCTGAAACTTCCAA-3′. The FRET probes used to detect the HAdV-B11 hexon gene have the following sequences: prbAdV11-Fluor, CACG TAACAGAAGAGGAAACCAATACTACTACTTA-Flr, and prbAdV11-705, LC-Red-705-CTTTTGGCAATGCTCCTGTAAAAGCTGAAGC-Phos. The primers used to detect the HAdV-B14 amplicon were prAd14-hex2F, 5′-GGGTTGAAA CTACTGAAGAACG-3′, and prAd14-hex2R, 5′-CATCTGTATCAGTCCACG ATT-3′. The FRET probes used to detect the HAdV-B14 hexon gene have the following sequences: prbAdV14-Flr, CAGTAAAAGCCGATGCTGACATTAC AA-Flr, and prbAdV14-705, LC-Red-705-GACGGACTACCAATAGGTTTG GAAGTCCC-Phos. The primers used to detect the HAdV-B21 amplicon were prAd21-hexF, 5′-TTGGACAAGGAAATCTCTTTGC-3′, and prAd21-hexR, 5′-T TACGTAGGTATCCACCAGG-3′. The FRET probes used to detect the HAdV-B21 hexon gene have the following sequences: prbAdV21-Flr, CACTCTTCCAAC TAACACCAACACTT-Flr, and prbAdV21-640, LC-Red-640-GACTACATGAAT GGGCGGGTGGTTC-Phos.

Experiments were performed using a Joint Biological Agent Identification and Diagnostic System (JBAIDS) and the LightCycler 2.0 (Roche). All JBAIDS experiments were performed using the same primers, probes, and conditions as those used with the LightCycler 2.0. The JBAIDS thermocycler was conceived and developed to rapidly identify biological warfare agents and other pathogens of concern for the U.S. military. For real-time JBAIDS PCR, cycling was carried out using a JBAIDS real-time thermocycler (Idaho Technologies, UT) using 1 μl (~1.8 μl of throat swabs) of extracted DNA in 2 μl of LC FastStart DNA Master HybProbe mix (Roche, Indianapolis, IN), containing 5 mM $MgCl_2$ and 400 nM forward and reverse primers. Reaction conditions were as follows: initial denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 1 s, annealing at 60°C for 15 s, and extension at 72°C for 5 s. The progress of real-time fluorescent PCR was monitored at 530 nm. To establish external standard curves for the quantification of each HAdV, genomic DNA from each strain was diluted in a 10-fold series (10^2 to 10^7 copies per reaction) and analyzed with the new assay. The samples that defined the standard curve were performed in triplicate and repeated twice.

Statistical analysis. To assess the differences in viral loads among HAdV-B14, HAdV-B3, HAdV-E4, AHdV-B7, and HAdV-B21, a nonparametric one-way analysis of variance model was used. If a significant difference was found, multiple comparison tests were computed to determine which viruses differed. A significance level with P at <0.05 was used for all tests.

RESULTS

Singleplex quantitative PCR. The detection of human adenoviruses B3, E4, B7, B11, B14, and B21 by conventional and real-time PCR from uncultured clinical throat swab specimens is summarized in Table 1. We tested 10, 20, 21, 5, 20, and 20 clinical specimens that were positive for HAdV-B3, -E4, -B7, -B11, -B14, and -B21, respectively. We also tested 20 clinical specimens that previously tested negative for HAdV by conventional PCR from patients with acute respiratory disease (ARD) in the same populations (Table 1). All samples were tested using the LightCycler 2.0 and the JBAIDS platforms. All positive samples were correctly identified except for three HAdV-B7-positive samples (Table 1). One of the 20 samples that tested negative for all HAdVs by conventional PCR tested positive for HAdV-E4 (Table 1). We sequenced and confirmed this sample as HAdV-E4.

TABLE 1. Summary of HAdV detection in clinical specimens from the NHRC by conventional PCR and real-time PCR using the LightCycler 2.0 and JBAIDS

Virus	No. of samples obtained using:					
	NHRC standard PCR		LightCycler 2.0 real-time PCR		JBAIDS real-time PCR	
	Positive	Negative	Positive	Negative	Positive	Negative
HAdV-B3	10	0	10	0	10	0
HAdV-E4	20	0	21	0	21	0
HAdV-B7	21	0	18	3	18	3
HAdV-B11	5	0	5	0	5	0
HAdV-B14	20	0	20	0	20	0
HAdV-B21	20	0	20	0	20	0
Negative by all six assays		20	0	19		19

Lower limits of detection for singleplex assays. Six standard curves were created using different dilutions of genomic DNA ranging from 50 to 10^8 genome copies per assay from each HAdV to determine the concentration of adenovirus in each clinical sample (Fig. 1). Using the LightCycler 2.0, the lower limits of detection for our singleplex assays were 50, 500, 500, 50, 50, and 50 genomic copies per reaction for HAdV-B3, -E4, -B7, -B11, -B14, and -B21, respectively (Table 2). We defined the lower limit of detection as the last dilution before the crossing points (CPs) ceased to increase. Singleplex assays for HAdV-B3, HAdV-E4, HAdV-B7, and HAdV-B11 were less sensitive using the JBAIDS (Table 2). Linear regression of the CP values and the quantity of genomic DNA revealed negative linearity for all curves (Fig. 1), corresponding to 98, 93, 88, 93, 92, and 97% PCR efficiency for singleplex assays for HAdV-B3, -E4, -B7, -B11, -B14, and -B21, respectively. The dynamic

TABLE 2. Lower limits of detection for HAdV singleplex reactions

Virus	Lower limits of detection (no. of genome copies/reaction)	
	LightCycler 2.0	JBAIDS
HAdV-B3	50	100
HAdV-E4	500	10^4
HAdV-B7	500	1,000
HAdV-B11	50	100
HAdV-B14	50	50
HAdV-B21	50	50

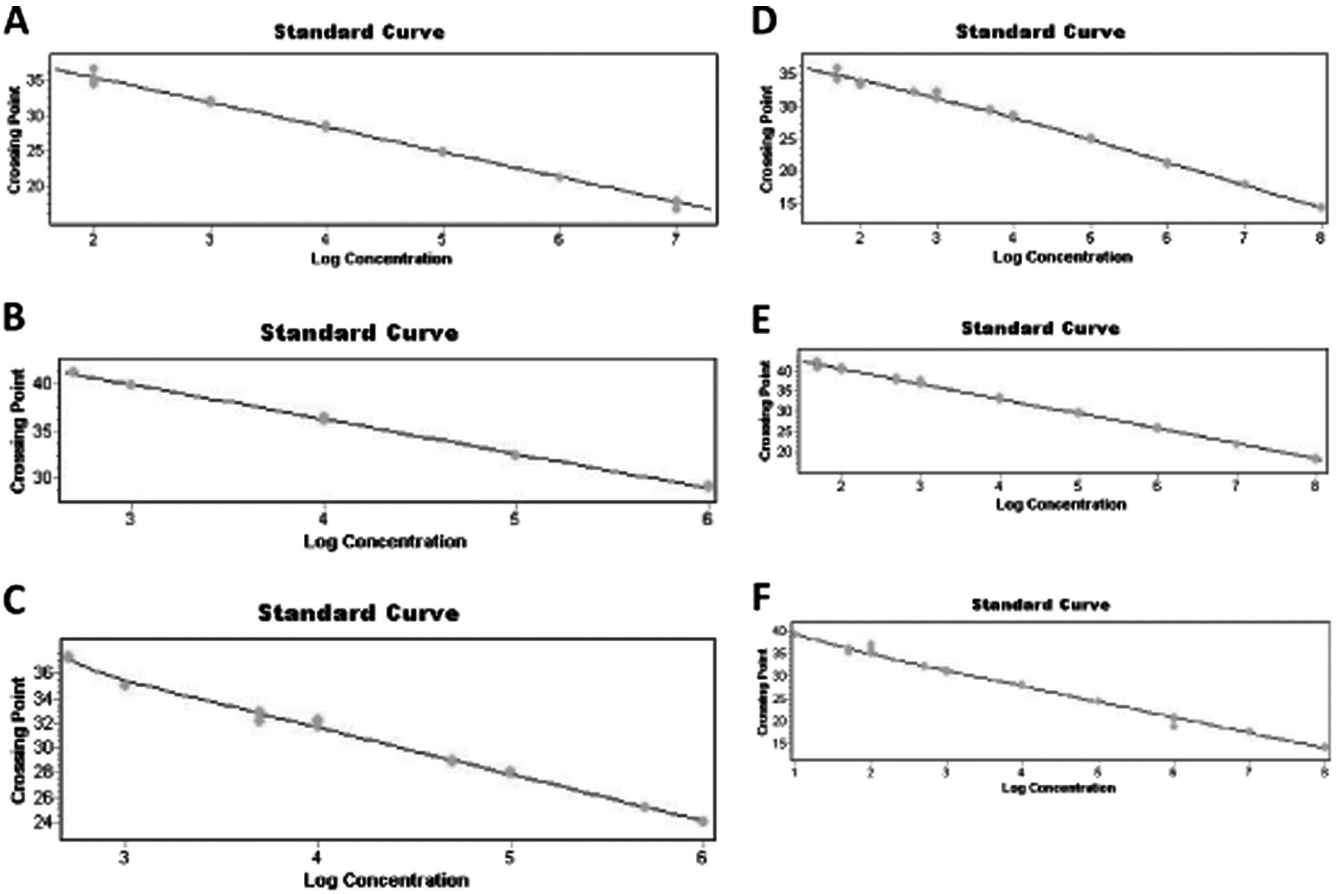

FIG. 1. Sensitivity of HAdV-B3, -E4, -B7, -B11, -B14, and -B21 detection by real-time LightCycler PCR in a series of dilutions of genomic HAdV-B3, -E4, -B7, -B11, -B14, and -B21 DNA. Linear regression of the standard curves for HAdV-B3 (A), HAdV-E4 (B), HAdV-B7 (C), HAdV-B11 (D), HAdV-B14 (E), and HAdV-B21 (F). We defined the lower limit of detection as the last dilution before the crossing point ceased to increase. For example, since the CPs for human adenovirus 11 at 50 and 10 copies were the same, we chose 50 as the lower limit of detection, because this is where the standard curve flattens.

TABLE 3. Ranges of viral loads in NHRC throat swabs using the LightCycler 2.0

Sample	Viral load range	Avg viral load of throat swabs (no. of genomic copies/ml)
HAdV-B3	2.0×10^7 to 1.5×10^9	5.6×10^8
HAdV-E4	5.0×10^5 to 1.0×10^9	2.1×10^8
HAdV-B7	6.0×10^6 to 3.6×10^8	1.7×10^8
HAdV-B14	6.9×10^5 to 4.5×10^8	2.0×10^7
HAdV-B21	1.4×10^6 to 5.9×10^8	1.2×10^8

range for the HAdV-B3, -B11, -B14, and -B21 assays was 50 to 1.0×10^8 DNA genomic copies per PCR. The dynamic range for the HAdV-E4 and -B7 assays was 5×10^2 to 1×10^8 DNA genomic copies per PCR. Using the same HAdV-B3-positive samples, we found that the CP values were lower using the JBAIDS in comparison to the CP values generated using the LightCycler 2.0. These experiments were repeated with similar results. Moreover, we found a statistically significant difference between the CP values for the HAdV-B3-positive specimens with the JBAIDS (mean, 27.8 ± 2.1) versus the LightCycler 2.0 (mean, 22.4 ± 2.2) ($P < 0.001$). The viral loads in analyzed throat swab samples varied from less than 5×10^5 to 1.5×10^9 per milliliter (Table 3). The viral loads for HAdV-B14 were 1 to 3 logs lower than the viral loads for the other viruses (Table 3). A statistically significant difference was found between the viral loads for HAdV-B14 and those for HAdV-B3, -E4, -B7, and -B21 ($P < 0.001$).

Multiplex real-time PCR assays. Singleplex assays for HAdV-B3 and HAdV-B11, HAdV-E4 and HAdV-B7, and HAdV-B14 and HAdV-B21 were combined to create three multiplex assays for use with clinical samples. Using the LightCycler 2.0, the HAdV-B3/B11, HAdV-E4/B7, and HAdV-B14/B21 multiplex assays were 100% concordant with the singleplex data generated from clinical specimens (Table 4). To simulate coinfected samples, we combined single infected samples that were positive for HAdV-B3 and -B11, HAdV-E4 and -B7, and HAdV-B14 and -B21. Our multiplex assays successfully detected 5 of 5, 18 of 18, and 19 of 19 artificially coinfected samples (Table 5).

Lower limits of detection for multiplex real-time PCR assays. Compared to the singleplex assays tested using the LightCycler 2.0, the HAdV-B3/B11, HAdV-E4/B7, and HAdV-B14/B21 multiplex assays were identical in their ability to detect HAdV-B3, -E4, -B7, -B11, and -B21 (Table 4). Moreover, when combined, only the HAdV-B14 and -B21 assays were less sensitive than the respective singleplex assays (100 versus 50 genomic copies per reaction) (Table 6). In contrast to the results generated using the LightCycler 2.0, with the exception of HAdV-B14 detection, the multiplex assays had much higher limits of detection with the JBAIDS (Table 6).

TABLE 4. Summary of human adenovirus detection in clinical specimens by multiplex real-time PCR using the LightCycler 2.0

Virus	Multiplex real-time PCR results (no. of positive samples)					
	HAdV-B3/B11		HAdV-E4/B7		HAdV-B14/B21	
	HAdV-B3	HAdV-B11	HAdV-E4	HAdV-B7	HAdV-B14	HAdV-B21
HAdV-B3	10					
HAdV-E4			21			
HAdV-B7				18		
HAdV-B11		5				
HAdV-B14					20	
HAdV-B21						20

TABLE 5. Summary of human adenovirus detection in artificially coinfected samples by multiplex real-time PCR using the LightCycler 2.0

Virus	Multiplex real-time PCR results (no. of positive samples/total no. of samples)					
	HAdV-B3/B11		HAdV-E4/B7		HAdV-B14/B21	
	HAdV-B3	HAdV-B11	HAdV-E4	HAdV-B7	HAdV-B14	HAdV-B21
HAdV-B3	5/5					
HAdV-E4			18/18			
HAdV-B7				18/18		
HAdV-B11		5/5				
HAdV-B14					19/19	
HAdV-B21						19/19

Specificity of quantitative PCR assays. Because adenoviruses are well conserved in the hexon and fiber genes, we wanted to determine if our assays were specific to the target HAdV types. Analytical specificity data are shown in Table 7. There was no cross-reactivity between adenovirus assays with different adenovirus types within species HAdV-B (types B3, B7, B11, B14, and B21), HAdV-E4, or representatives from other HAdV species (B16, A12, D22, F40, and G52). In addition, our real-time PCR assays did not generate false-positive results when challenged with genomic DNA extracted from other agents which cause respiratory disease such as *Haemophilus influenzae*, influenza A virus, human rhinovirus, human parainfluenza virus, human respiratory syncytial virus, *Chlamydophila pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Mycoplasma pneumoniae*, and *Legionella pneumophila*. Additionally, when we tested all samples with the multiplex assays, we did not see any cross-reactivity (data not shown).

DISCUSSION

For many years, there has been a need for a rapid, accurate diagnostic test for HAdV-B3, -E4, -B7, -B11, -B14, and -B21. In crowded adult populations subject to high-attack-rate epidemics of adenovirus, and in light of the variable severity of outbreaks seen with specific HAdV types and the availability of type-specific vaccines, it is critical to not only be able to detect

TABLE 6. Lower limits of detection for HAdV LightCycler multiplex reactions

Virus	Lower limit of detection (no. of genome copies/PCR)	
	LightCycler 2.0	JBAIDS
HAdV-B3/B11	50/50	$10^6/10^4$
HAdV-E4/B7	500/500	$10^6/10^4$
HAdV-B14/B21	100/100	$10^3/100$

TABLE 7. Specificity of each HAdV assay

Pathogen	Assay specificities					
	HAdV-B3	HAdV-E4	HAdV-B7	HAdV-B11	HAdV-B14	HAdV-B21
HAdV-B3	+					
HAdV-E4		+				
HAdV-B7			+			
HAdV-B11				+		
HAdV-B14					+	
HAdV-B16						
HAdV-B21						+
HAdV-A12						
HAdV-D22						
HAdV-F40						
HAdV-G52						
Haemophilus influenzae						
Influenza A virus						
Human rhinovirus						
Human parainfluenza virus						
Human respiratory syncytial virus						
Chlamydophila pneumoniae						
Escherichia coli						
Klebsiella pneumoniae						
Pseudomonas aeruginosa						
Mycoplasma pneumoniae						
Legionella pneumophila						

all common adult respiratory HAdV types but to also discriminate between them. We describe the first multiplex real-time PCR assay for the detection, discrimination, and quantification of HAdVs which cause ARD in military recruits, using uncultured clinical samples.

Adenovirus infection has been a recognized problem at military training centers since its discovery in the 1950s (14). HAdV-E4 was the predominant adenovirus (98% of isolates) at recruit training facilities from 1996 through 2005. In 2006, HAdV-B14a emerged as a significant contributor to acute respiratory disease (ARD) and severe pneumonias in several U.S. cities and at military recruit training centers across the United States, the first time this type had been isolated in the Western Hemisphere (14, 22). At the same time, several previously common types reemerged and caused significant outbreaks. These included HAdV-B3, -B7, and -B21, all of the adult respiratory types ever identified as the primary etiological agent of a recruit camp outbreak. Given this diversity, it is important to have high-quality diagnostic tools readily available that can determine which adenovirus is causing an outbreak.

Interestingly, the viral loads for HAdV-B14 that we detected in uncultured clinical specimens were 1 to 3 logs below those seen in our laboratory for types HAdV-B3, -E4, and -B21 (Table 3). One possibility for the differences in viral loads is that the samples from patients infected with HAdV-B14 were acquired at different time points (e.g., before or after peak viremia) than the samples from patients infected with other HAdVs. However, all patient specimens were collected when ill recruits self-reported for medical care, so there is no reason to suspect any such bias. Another possibility for the lower viremia seen in patients infected with HAdV-B14 may be that type-specific viral factors act to generate lower-titer infections. This offers further purpose for the development of real-time assays, as these assays are generally more sensitive than standard PCR assays or rapid antigen tests (10, 23). The capability to quantitate viral loads will be a valuable tool to further our understanding of type-specific aspects of adenoviral disease.

A major difficulty in designing nucleic acid tests for adenoviruses is that there is a dearth of genetic information outside the hexon and fiber genes. Designing an assay for HAdV-B7 proved to be quite difficult, because there are few differences between the HAdV-B3 and -B7 hexon genes. The rationale for using the hexon gene as a target sequence is severalfold. First, this region includes the primary antigenic determinant. Second, all HAdV types can be distinguished by unique sequences in this region (20), and the hypervariable regions of the hexon provide the most sequence diversity for the generation of type-specific primers (Table 8). Because of the value of this region for discrimination, there is much more data available in GenBank for this locus than for the rest of the genome. For example, 49 of the 100 HAdV-B7 sequences in GenBank were from the hexon gene. Using another gene as a target would have increased the risk of detecting more than one type of adenovirus, as there is little information known about other genes outside this area.

Four out of six singleplex assays had lower limits of detection using the LightCycler 2.0 (Table 2). A noteworthy point was that our real-time PCR assay detected HAdV-E4 in a previously negative sample (which we confirmed by sequencing), demonstrating the sensitivity of our assay. Unfortunately, the multiplex assays cannot be used in the JBAIDS due to a lack of sensitivity. This may be due to the fact that the air that heats/cools the glass capillaries in the JBAIDS is allowed to mix with the air outside the machine, which does not happen with the LightCycler 2.0.

Our assays can be used to determine whether or not a sample is positive or negative for ARD-causing adenoviruses in less than 2 h. With the exception of HAdV-B7, our real-time PCR assays demonstrated 100% concordance with the conven-

TABLE 8. Primers for real-time PCR amplification of adenovirus

Virus	Primer/probe	Gene	Position in genome	Sequence	Amplicon size (bp)
HAdV-B3	prAdV3-FibF	Fiber	32073	CGTATCCATTTGTCCTTCCTAAT	153
	prAdV3-FibR		32225	TAACATAGGATGTGCGACTATCT	
	prbAdV3-Flr		32156	GCCCTTTTTCCGTTGGAAGTTACTG-Flr	
	prbAdV3-640		32183	LC-Red-640-ATGCTTAATAAACGCC-Phos	
HAdV-E4	prAd4-470F	Fiber	32118	ATGATAAAGGGAATATAAAGATTACCCTAA	160
	prAd4-629R		32777	GTACTACTGGTTCCGAACTC	
	prbAd4-FLR538		32186	AGCAACATCAGTTGGGCTAAAGGTATAA-Flr	
	prbAd4-640		32216	LC-Red-640-TTTGAAGATGGTGCCATAGCTACAAACATTGGT-Phos	
HAdV-B7	prHAdV7-1230F	Hexon	19895	CAAATATCAAGGCATTAAACCTAGAG	158
	prHAdV7-1396R		20061	CATCTGGAAGGTACAAAGCC	
	prbAdV7-Fluor9		19972	CCATAGGCAACAATCTGGCTATGGAAATTA-Flr	
	prbAdV7-705-9		19998	LC-Red-705-ATCCAAGCTAATCTTTGGAGAAGTTTTCTGTACT-Phos	
HAdV-B11	prAD11-5F	Hexon	18655	GTGGATTGCAGAAGGTGTA	165
	prAD11-5R		18819	TTACTTTCTTCATCTGAAACTTCCAA	
	prbAdV11-Fluor		18695	CACGTAACAGAAGAGGAAACCAATACTACTACTTA-Flr	
	prbAdV11-705		18732	LC-Red-705-CTTTTGGCAATGCTCCTGTAAAAGCTGAAGC-Phos	
HAdV-B14	prAd14-hex2F	Hexon	18667	GGGTTGAAACTACTGAAGAACG	225
	prAd14-hex2R		18891	CATCTGTATCAGTCCACGATT	
	prbAdV14-Flr		18745	CAGTAAAAGCCGATGCTGACATTACAA-Flr	
	prbAdV14-705		18774	LC-Red-705-GACGGACTACCAATAGGTTTGGAAGTCCC-Phos	
HAdV-B21	prAd21-hexF	Hexon	19799	TTGGACAAGGAAATCTCTTTGC	201
	prAd21-hexR		19999	TTACGTAGGTATCCACCAGG	
	prbAdV21-Flr		19920	CACTCTTCCAACTAACACCAACACTT-Flr	
	prbAdV21-640		19948	LC-Red-640-GACTACATGAATGGGCGGGTGGTTC-Phos	

tional PCR assays initially used to identify HAdV-B3, -E4, -B14, and -B21 in clinical samples. These assays provide quantitative data and do not cross-react with other ARD-causing viruses or bacteria (Table 6). When future HAdV outbreaks occur within the Department of Defense or within the civilian community, similar to the outbreak at Lackland AFB, we will have the tools available to rapidly determine which adenovirus is causing the infection.

We generated single- and multiplex real-time PCR assays that are sensitive and specific and provide accurate quantitation of HAdVs, which cause respiratory disease. These assays will be useful as routine diagnostic tools for the rapid detection of clinical samples using already existing platforms which are positive for HAdV-B3, -E4, -B7, -B11, -B14, and -B21.

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